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Kinetic Schemes for Post-Synchronized Single Molecule Dynamics
Chunlai Chen, Joseph M. Laakso, E. Michael Ostap, **Yale E. Goldman**, Henry Shuman.
University of Pennsylvania, Philadelphia, PA, USA.
Recordings of force, fluorescence, or ionic membrane current from single molecules show directly their individual trajectories, but many events need to be aggregated to obtain reaction paths and mean kinetic parameters. A procedure termed 'post-synchronization' has been developed to combine individual interactions that are either noisy or complicated by successive states that have similar signals. To create a post-synchronized ensemble, many trajectories are temporally lined up at an identifiable 'trigger' transition and averaged revealing the dynamics of the process leading up to and following the synchronized transition. Averaged recordings following a trigger have the same properties as an ensemble perturbation experiment, i.e., rate constants can be determined similarly to temperature jump or rapid mixing experiments. However, determining rate constants from averaged recordings leading up to aligned trigger transitions is not as straightforward if the reaction has reversible steps or branches. The averaged traces have properties of a time-reversed kinetic scheme. A general method to determine rate constants for the ''reversed time'' scheme leading up to the trigger is the following: (0) Add a reaction to complete the enzymatic cycle, if necessary. (1) Solve the relative steady state concentrations. (2) Calculate the transition rates for the time-reversed scheme from $P(A_i \mid B_{i+1}) = P(A)\ P(B_{i+1} \mid A_i) / P(B)$, where P(A) and P(B) are steady-state occupancies, $P(B_{i+1} \mid A_i)$ is a normal forward transition probability of entering state B from state A, and $P(A_i \mid B_{i+1})$ is the time-reversed transition probability that it was in A given it's in B at a later time. (3) Estimate the kinetics of the ''time-reversed'' perturbation experiment using the rate constants determined in step (2). Supported by NIH grants P01-GM087253 and R01-GM080376.

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Three-Dimensional Dynamical Observations of Nanocolloid in Water using Diffracted Electron Tracking
Naoki Ogawa[1,2], Kentaro Hoshisashi[2,3], Hiroshi Sekiguchi[2,3], Yasuhisa Hirohata[1,2], Akira Ishikawa[1,2], Yuji C. Sasaki[2,3].
[1]Department of Physics, College of Humanities and Sciences, Nihon University, Tokyo, Japan, [2]CREST SASAKI-team, Japan Science and Technology Agency, Osaka, Japan, [3]Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan.
Diffracted X-Ray tracking (DXT) method has been developed for obtaining dynamical information at single protein molecule level (1). The motion of an individual single gold nanocrystal can be observed by this method, which is linked to specific site in the molecule, using a time resolved Laue diffraction technique. This method needs a very strong X-ray source, so we continue to develop a compact instrument with use of the electron beam instead of the X-ray. The Electron Back-Scattered Diffraction (EBSD) is adopted to monitor the Three-dimensional (3D) crystal orientation of the gold nanocrystal. We call this the Diffracted Electron Tracking (DET). For this purpose, we have developed the wet cell using the very thin carbon sealing film for maintained atmospheric pressure from high vacuum surroundings of a scanning electron microscope (SEM), and confirmed that the EBSD pattern (EBSP) can be obtained from the gold nanocrystals through the carbon sealing film of the wet cell. When the gold nanocrystal moved by the motion of labeled molecules, the change of the EBSP is observed. Using DET, we measured the motion of gold nanocrystal that labeled on silane-coupling-agent under different conditions; in water, in argon gas or vacuum environments. From results, the motions of gold nanocrystals in water environment were over one hundred times as large as in argon gas environment. And in vacuum environment, few motions were observed. We will discuss the difference between DXT and DET at observed gold nanocrystal motions in water environment. Recently, we confirmed EBSD can obtain from 40nm diameter of commercial colloidal gold. This finding may contribute to develop as a very general single molecular technology.
1. Global twisting motion of single molecular KcsA potassium channel upon gating.
H. Shimizu et al. Cell. 2008 Jan 11;132(1):67-78.

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Localized Heating of Single Oligonucleotides using Infrared Light
Erik D. Holmstrom, David J. Nesbitt.
NIST, JILA, and University of Colorado, Boulder, CO, USA.
Fluorescence spectroscopy is an exceptional tool that provides the ability to ''observe'' the various motions associated with biomolecular dynamics. The temperature-dependence of these dynamical motions can provide rich thermodynamic information about the observed process. To obtain superior temporal (< 2 ms) and spatial (< 20 μm) control of temperature, along with a greater range of accessible steady-state temperatures (20-90 °C), we have developed an infrared absorption based heating system to locally heat a small volume of water via absorption in the second overtone of the OH stretch (1445 nm). With the infrared light focused onto the confocal volume of a single-molecule fluorescence microscope the aqueous solution can be heated rapidly and locally. The IR heating system primarily functions as a steady-state source of heat for temperature-dependent kinetic studies of biomolecular conformational changes. However, given the relatively fast temporal control of laser intensity we have utilized short pulses of heat (10-1000 ms) to study the dissociation of a DNA duplex as well at the unfolding of an RNA pseudoknot at the single-molecule level.

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In Situ Quantitative Imaging of Single-Molecule Co-Immunoprecipitation
Hong-Won Lee[1], Jang-Hyun Yoo[1], Byung-San Choi[1], Han-Ki Lee[2], Tae-Young Yoon[1].
[1]KAIST, 335 Gwahak-ro, Yuseong-gu, Daejeon, Korea, Republic of, [2]University of California, San Francisco, San Francisco, CA 94115, CA, USA.
Co-immunoprecipitation (co-IP) and western blotting are the gold standards for assessing protein-protein interactions.Their protein band output, however, only provides qualitative and static information on protein compositions and their interactions. In this study, we demonstrate a quantitative transformation of the traditional western and co-ip techniques using single-molecule level in situ measurements with millisecond time resolution. Using these modified techniques, we have quantified the molar concentration of specific proteins in a whole cell extract and determined the stoichiometry and kinetics of their interactions. We have used our methods to quantify the expression level and signaling frequency of oncogenic Ras that has been directly pulled down from cancerous cells. Our results also suggest that these techniques will make valuable additions to the molecular diagnostic repertoire of modern biomedicine.

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Sub-Millisecond Single Molecule Fluorescence Imaging Combined with Dual Optical Tweezers on DNA Tethers
Isabel Llorente Garcia, Oliver L.J. Harriman, Mark C. Leake.
Oxford University, Oxford, United Kingdom.
We present a novel optical design which simultaneously combines multi-color fluorescence microscopy with single molecule sensitivity and sub-millisecond temporal resolution, together with the accurate positioning control and diagnosis of optical tweezers.
This versatile imaging set-up, currently under development, will serve a number of DNA tether experiments in which the DNA will be held between two laser-trapped beads and both the tether and a variety of bound protein complexes will be visualised simultaneously.
The set-up includes three simultaneous visible laser light excitation paths of different wavelengths, each with the flexibility of switching between total-internal reflection (TIRF), confocal or slimfield [1] microscopy configurations. Time-shared laser optical tweezers give rise to two simultaneous optical traps for the confinement of the beads attached to the ends of the DNA filament providing accurate control of their positions.
As a proof-of-principle, our design enables simultaneous trapping of beads with optical tweezers in dual traps separated by distances of the order of ca. 10μm, as well as imaging of bright fluorescent beads at sub-ms capture rates.
[1] R. Reyes-Lamothe, D. J. Sherratt and M. C. Leake. Stoichiometry and architecture of active DNA replication machinery in Escherichia coli. *Science.* 328, 498-501 (2010).

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Measurements of the Trap Stiffness of Optical Tweezers
Dong-yun Lee, Chung-il Ha, Hyuk-Kyu Pak.
Pusan National University, Busan, Korea, Republic of.
When using the optical tweezers, determing the exact value of the trap stiffiness is very important. In this work, displacements of micron size particle were measured as a function of time by using two different methods; the passive method which measures the trap stiffness of the trapped particle at a fixed position by optical tweezers and the active method which measures the trap stifffness by forcefully oscillating the trapped particle. When using the passive method,